

# Protection Against Acute Lung Injury by Intravenous or Intratracheal Pretreatment with EPI-HNE-4, a New Potent Neutrophil Elastase Inhibitor

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Excessive accumulation of active neutrophil elastase (NE) in pulmonary fluids and tissues of patients with cystic fibrosis (CF) is thought to act on the lungs, compromising their structure and function. The aim of this study was to investigate the *in vitro* and *in vivo* protective effect of a new, rapidly acting, potent ( $K_i = 5.45 \times 10^{-12}$  M and  $K_{on} = 8 \times 10^6$  M<sup>-1</sup>s<sup>-1</sup>) and specific human NE inhibitor, EPI-HNE-4, engineered from the Kunitz domain. The results demonstrated that this inhibitor was able to (i) effectively inhibit *in vitro* the high levels of active NE present in a medium as complex as sputum from children with CF, with a measured IC<sub>50</sub> equal or close to the calculated IC<sub>50</sub> in 60% of cases, and (ii) almost completely block (91%) the N-formyl-methionine-leucine-phenylalanine-induced migration of purified human neutrophils across a Matrigel basement membrane. Intratracheal administration (250, 175, or 100 μg per rat) of the inhibitor 5 min before instillation of pure human NE (HNE) (150 μg per rat) to rats induced effective, dose-dependent protection of the lungs, 4 h later, from hemorrhage, serum albumin leakage, residual active NE, and discrete neutrophil influx in air spaces induced by instillation of pure HNE. Intravenous administration (3 mg per rat) of EPI-HNE-4, 15 min before instillation of the soluble fraction of pooled sputum (delivering 120 μg of active NE per rat) from children with CF, effectively reduced (64%), 4 h later, the massive neutrophil influx induced by sputum instillation. Overall, these data strongly suggest that associated aerosol and systemic administration of EPI-HNE-4 would be beneficial in the treatment of CF.

Protease-antiprotease imbalance has been known for a long time to be involved in a variety of inflammatory lung diseases, such as chronic obstructive pulmonary disease, adult respiratory distress syndrome (1, 2), and cystic fibrosis (CF). More particularly, apart from high levels of matrix metalloproteinase MMP-9 (also called gelatinase B or 92-kD gelatinase) (3), elevated levels of active neutrophil elastase (NE), a 29-kD serine protease, have often been found in sputum from children with CF. Importantly, active NE has been measured in lung fluids from children as young as 1 yr (4), has been found in pulmonary secretions in the majority of children with CF by the age of 7 yr and in virtually all adults with CF (5–6), and has been correlated with the degree of severity of the disease. This protease, stored in the primary granules of neutrophils, is released after phagocytosis, membrane damage, or cell lysis.

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Abbreviations: bronchoalveolar lavage, BAL; cystic fibrosis, CF; N-formyl-methionine-leucine-phenylalanine, FMLP; human neutrophil elastase, HNE; neutrophil elastase, NE; secretory leukoprotease inhibitor, SLPI.

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Excessive accumulation of NE in pulmonary fluids and tissues of patients with CF is thought to compromise lung structure and function because NE has been reported to cause epithelial damage (7), lung hyperpermeability (8), mucus hypersecretion (9–10), mucous gland metaplasia (11), and mucociliary dysfunction (12–13). Overall, NE is able to degrade most of the macromolecular components of the pulmonary extracellular matrix, including elastin, type III and type IV collagens, fibronectin, the core proteins of proteoglycans, and laminin, and, when in excess, it actively participates in the destruction of lung structures. NE may also induce further inflammation by generating chemotactic peptides (14) and initiating the production of chemotactic cytokines (15–16), thus perpetuating cycles of inflammation and destruction of airway walls leading to the development of bronchiectatic cysts.

The presence of high levels of free NE in the air spaces indicates a defect of α1-proteinase inhibitor and secretory leukoprotease inhibitor, and this defect has led several authors to propose treatment by protease inhibitor. More particularly, the secretory leukoprotease inhibitor (SLPI) found in serous cells of tracheal and bronchial submucosal glands and in nonciliated cells of the bronchial and bronchiolar epithelium provides the natural major anti-NE protective screen of the epithelial surface of the upper respiratory tract (17). Some studies have considered the use of SLPI to enhance the anti-NE protective screen of the pulmonary epithelium as a therapeutic approach to disorders associated with NE-mediated pulmonary damage. Vogelmeier and colleagues have shown that aerosolized recombinant SLPI directly increases the anti-NE capacity of the lung of mixed-breed sheep, particularly on the pulmonary epithelial surface (18), whereas Susuki and colleagues demonstrated protection by intravenous pretreatment with recombinant half-length SLPI against airway constriction and hyperresponsiveness induced by human NE (HNE) aerosolization in guinea pigs (19). The work by Rees and colleagues (20) has also demonstrated that NE in CF airway secretions causes lung tissue damage and that rats can be protected from such damage by an orally available monocyclic β-lactam inhibitor of NE. When injected intravenously, another NE inhibitor, NO-5046, or N-(2-[4-(2,2-dimethylpropionyl-oxy)phenylsulfonamino]benzoyl) amino acetic acid, was found to attenuate lipopolysaccharide-induced acute lung injury in guinea pigs (21).

The aim of this study was to investigate the *in vitro* and *in vivo* protective effect of a new potent and specific HNE inhibitor, EPI-HNE-4. This highly specific and potent inhibitor (6,237 Da) of HNE was discovered and engineered using the patented phage-display technology “directed

evolution of novel binding protein" (22). It was derived from the second Kunitz-domain of the light chain of the naturally occurring human proteinase inhibitor (inter  $\alpha$  inhibitor). It is 50-fold more potent than the most potent Kunitz derivative HNE inhibitor described, and its affinity for HNE is approximately  $10^6$ -fold higher than that of bovine trypsin inhibitor or SLPI. EPI-HNE-4 is a rapid-acting and potent inhibitor of HNE ( $K_i = 5.45 \times 10^{-12}$  M, and  $K_{on} = 8 \times 10^6$  M $^{-1}$  s $^{-1}$ ) and is resistant to oxidants such as chloramine T in contrast with SLPI and  $\alpha$ 1-proteinase inhibitor. It is also stable in the presence of acid pH or high temperature.

To evaluate the capacity of EPI-HNE-4 to access the HNE catalytic site in a medium as complex as sputum from children with CF, we measured the *in vitro* protective effect of EPI-HNE-4 on HNE by incubating it with the soluble aqueous fraction of sputum or whole sputum. We have previously demonstrated that human neutrophil migration across the basement membrane involves NE (23); here, we also evaluated the level of EPI-HNE-4-induced inhibition of transmigration of chemoattracted human neutrophils across a Matrigel membrane. Finally, we evaluated the *in vivo* protective effect of intravenous or intratracheal administration of EPI-HNE-4 on the observed lung injury induced by either pure HNE or by soluble fraction of CF sputum containing similar amounts of active NE.

## Materials and Methods

### Preparation of NE and Soluble Sputum Fractions

Whole sputum was collected from patients (mean age, 12 yr; range, 1–23 yr) with advanced CF hospitalized (Service de Pédiatrie, Centre Hospitalier Intercommunal de Créteil, Service de Pneumologie et d'Allergologie Pédiatrique, Hôpital des Enfants Malades, Paris; Service de Gastro-Entérologie-Nutrition Pédiatrique Hôpital Robert Debré, Paris, France) for acute pulmonary exacerbation just before starting antibiotic therapy and steroids. Steroid therapy was used only in a few patients with severe respiratory distress.

The *in vitro* studies evaluating the inhibitory capacity of EPI-HNE-4 on active HNE secreted in excess in the sputum of seven children with CF were performed by using whole sputum or soluble (sol) fraction of CF sputum from each child. Whole sputum was stored on ice for up to 3 h and then vortexed and fractionated into soluble and gel fractions by centrifugation at  $8,000 \times g$  for 20 min.

The *in vivo* studies evaluating the protective effect of EPI-HNE-4 on lung injury induced by intratracheal instillation of the sol fraction of CF sputum were performed by using the pooled sol fraction of sputum from several children.

HNE, chromatographically purified from human sputum, was obtained from Elastin Products (Lausanne, Switzerland) and was stored as a 2 mg/ml stock solution in 0.15 M NaCl (pH 7.4) at 4°C.

### Evaluation of Inhibitory Capacity of EPI-HNE-4 on Active HNE Secreted in Sputum of Children with CF

HNE activity in sputum or soluble fraction of sputum was measured kinetically for 30 min using the synthetic substrate N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide (Sigma, St. Louis, Missouri) at a concentration of 0.42 mM, in 100 mM Tris HCl (pH 8), 0.15 M NaCl, 1 mM CaCl<sub>2</sub>, and 0.01% Brij 35. Changes in absorbance were measured spectrophotometrically at 410 nm. The  $K_m$  of this enzyme and substrate was approximately 0.08 mM. This value was close to the previously published value (0.14 mM) (24). The active site of elastase was verified by back titration using hu-

man recombinant SLPI (R&D Systems, Minneapolis, MN). Our results clearly showed that SLPI inhibited elastase activity at a 1:1 molar ratio of SLPI to elastase.

For each patient, the inhibitory capacity ( $IC_{50}$ ) of EPI-HNE-4 (the concentration of inhibitor at which the enzyme was inhibited by 50%) was evaluated in relation to the molar NE concentration in sputum or soluble fraction of sputum and was then compared with the same molar concentration of pure HNE to test the capacity of EPI-HNE-4 to access the HNE catalytic site in a medium as complex as sputum. The  $IC_{50}$  was evaluated under limiting conditions for  $K_m > 2$  (substrate saturating conditions). Before the evaluation of  $IC_{50}$ , the protease inhibitor was incubated for 15 min in the presence of whole sputum or soluble fraction of sputum to ensure inhibition. The NE protein concentration in sputum ( $\mu\text{g}/\mu\text{l}$ ) was determined on the basis of the activity measured on MeOSuc-Ala-Ala-Pro-Val-NA, using neutrophil elastase from Elastin Products as gold standard.

### Transmigration of Human Neutrophils Across Matrigel Membranes

Human neutrophils were isolated from heparinized whole blood obtained from healthy volunteers and processed as previously described (23). Briefly, neutrophils were obtained by dextran sedimentation, centrifugation through Ficoll-Hypaque, and hypotonic lysis of red cells. Preparations contained 95–97% neutrophils, which were > 98% viable by Trypan Blue exclusion. Isolated neutrophils were resuspended in HBSS with calcium and magnesium at a concentration of  $5 \times 10^6$  cells/ml.

Neutrophil chemotaxis was investigated by using a Falcon cell culture insert (Becton-Dickinson, Le Pont de Claix, France) containing an 8- $\mu\text{m}$  pore size membrane and a Matrigel Invasion Chamber (Becton-Dickinson). The Matrigel Invasion Chamber consists of a Falcon cell culture insert in which the porous membrane is coated with Matrigel basement membrane, a solubilized basement membrane extracted from Engelbreth-Holm-Swarm mouse sarcoma that contains laminin, collagen type IV, heparan sulfate proteoglycan, entactin, and growth factors. After rehydration of the basement membrane, a suspension containing approximately  $10^6$  neutrophils was added to the upper chamber, with or without  $10^{-6}$  M EPI-HNE-4. This molar concentration was chosen somewhat higher than the maximal molar concentration ( $0.6 \times 10^{-6}$  M) of NE able to be released by  $10^6$  neutrophils used for each chemotaxis experiment. The Hanks' balanced salt solution buffer (with calcium and magnesium) with or without  $10^{-7}$  M N-formyl-Met-Leu-Phe (FMLP) was added to the lower chamber. FMLP, a synthetic soluble bacterial product, is known to be one of the most potent chemoattractants for phagocytes. The devices were incubated at 37°C in 5% CO<sub>2</sub> for 3 h. After incubation, neutrophils on the upper surface of the basement membrane were removed with a cotton swab before Hematoxylin Harris staining. The deep surface of the membrane was then examined for neutrophil quantification using light microscopy. Each condition (without FMLP, with FMLP and without EPI-HNE-4, with FMLP and EPI-HNE-4) was investigated in triplicate for each healthy blood donor ( $n = 3$ ). Cell numbers were counted from 20 areas of each membrane selected at random, using a reticled eyepiece (magnification  $\times 400$ ).

### HNE-Induced and Sputum Sol-Induced Lung Injury: EPI-HNE-4 Inhibitor Treatment

Male Sprague Dawley rats (Iffa Credo, L'Arbresle, France) weighing 250–270 g were treated with pure HNE or soluble fraction of sputum by intratracheal injection under halothane anesthesia. Instillations were performed either at a constant amount of pure HNE (150  $\mu\text{g}/300 \mu\text{l}$  of saline solution) or at a constant volume of soluble fraction of sputum (250–300  $\mu\text{l}$  containing 100–120  $\mu\text{g}$  of active NE). Intravenous EPI-HNE-4 (3 mg/300  $\mu\text{l}$  per rat) was

administered 15 min before intratracheal instillation of pure HNE or soluble fraction of sputum, whereas intratracheal EPI-HNE-4 (250  $\mu\text{g}/200 \mu\text{l}$  per rat) was administered 5 min before pure HNE or soluble fraction of sputum. This high dose of EPI-HNE-4 was injected intravenously to compensate for its relatively rapid plasma clearance (half-life, 7 h) and to promote a potential molar excess of EPI-HNE-4 over HNE in the air spaces. The dose of EPI-HNE-4 injected intratracheally corresponded to an approximate 6-fold higher molar concentration in air spaces than that obtained by instilled HNE. EPI-HNE-4 was prepared in 50 mM sodium acetate, 100 mM sodium sulfate, 20% ethanol, pH 5.5 buffer. This preparation was 8-fold diluted in 0.15 M NaCl before intratracheal instillation. Four hours after HNE or sputum sol instillation, rats received an intraperitoneal injection of pentobarbital sodium; they were then exsanguinated, and their lungs were either fixed and embedded in paraffin or lavaged with  $4 \times 5$  ml washes of isotonic saline and then investigated for cell counts, hemorrhage, albumin content, and residual NE activity. The time of death (4 h after HNE or sputum sol instillation) was chosen according to previous studies investigating the time course of lung responses to instilled pure HNE or CF sputum sol in rats (20). Five series of experiments were therefore performed:

1. In the first experimental series, one group of 10 rats received an intravenous injection of 3 mg of EPI-HNE-4, followed by intratracheal instillation of 150  $\mu\text{g}$  of pure HNE 15 min later. Another group of 14 rats was instilled with pure HNE in the absence of NE inhibitor, and a third control group of 11 rats received an intravenous injection of EPI-HNE-4 followed by intratracheal instillation of saline solution 15 min later.
2. In the second experimental series, one group of 11 rats received an intratracheal instillation of 250  $\mu\text{g}$  of EPI-HNE-4, followed by instillation of 150  $\mu\text{g}$  of pure HNE 5 min later. Another group of 11 rats received an instillation of pure HNE without NE inhibitor, and a third control group of 6 rats received an instillation of EPI-HNE-4 followed by an instillation of saline solution 5 min later.
3. In the third experimental series, one group of 15 rats received an intravenous injection of 3 mg of EPI-HNE-4 followed by intratracheal instillation of 300  $\mu\text{l}$  of CF sputum-soluble fraction (containing 120  $\mu\text{g}$  of active NE) 15 min later. Another group of 11 rats received an instillation of CF sputum-soluble fraction without NE inhibitor, and a third control group of 11 rats received an intravenous injection of 3 mg of EPI-HNE-4 followed by instillation of saline solution 15 min later.
4. In the fourth experimental series, one group of 10 rats received an intratracheal instillation of 250  $\mu\text{g}$  of EPI-HNE-4 followed by intratracheal instillation of 250  $\mu\text{l}$  CF sputum-soluble fraction (containing 120  $\mu\text{g}$  of active NE) 5 min later. Another group of 11 rats received an instillation of CF sputum-soluble fraction without NE inhibitor, and a third control group of 6 rats received an instillation of EPI-HNE-4 followed by instillation of saline solution 5 min later.
5. A fifth experimental series was performed to investigate the dose-dependent inhibitory effect of intratracheally instilled EPI-HNE-4 on acute lung injury induced by pure HNE. In these series, three groups of 10 rats received instillations of 250, 175, and 100  $\mu\text{g}$  EPI-HNE-4, respectively, followed by instillation of 150  $\mu\text{g}$  of pure HNE 5 min later. These three EPI-HNE-4 doses corresponded to 6.6-, 4.6-, and 2.6-fold excess of HNE molar concentration compared with the pulmonary concentration, respectively. Another group of 10 rats received instillations of pure HNE alone, and a last group of 6 rats received instillations of 250  $\mu\text{g}$  of EPI-HNE-4 followed by instillation of saline solution 5 min later.

#### Analysis of Bronchoalveolar Lavage

Four fractions (5 ml) of physiological saline solution were used for bronchoalveolar lavages (BAL). All BAL samples obtained

from each rat were pooled ( $\sim 17.5$  ml) for analysis of cell counts and protein content. The cell count was evaluated from pooled BAL using a Malassez hemocytometer. A pooled BAL aliquot was also studied for released hemoglobin using the "total hemoglobin" kit (Sigma). The remaining pooled BAL was centrifuged at  $300 \times g$  for 10 min to pellet the cells.

The supernatant was used for analysis of albumin by measuring binding of bromocresol green and for analysis of residual NE activity by spectrophotometrically measuring changes in absorbance at 410 nm of the synthetic and specific substrate N-methoxy-succinyl-Ala-Ala-Pro-Val-paranitroanilide.

The cell pellet was analyzed for differential cell counts obtained from cytopspin preparations stained with Diff-Quick (Dade Behring, Paris, France). Red blood cell lysis was performed before evaluation of differential cell counts in all groups receiving instillations of pure HNE due to the presence of acute hemorrhage.

#### Histologic Studies

Lungs were inflated and fixed at a pressure of 20 cm  $\text{H}_2\text{O}$  by instillation of 2.5% formaldehyde via a polyethylene catheter inserted into the trachea. Lungs were then dehydrated, degassed, and embedded in paraffin. Sagittal sections cut from whole lungs were stained with orcein picro indigo carmine and processed for histologic studies.

#### Statistical Analysis of the Data

Results are presented as the mean and standard deviation, with  $n \geq 6$  for all groups. The effect of NE inhibitor treatment (e.g., comparing residual NE activity in response to instilled saline solution, instilled pure HNE, and instilled pure HNE in animals pretreated with EPI-HNE-4) was analyzed by two-way analysis of variance. Differences were considered to be significant when  $P < 0.05$ . Statistical procedures were performed with Statview statistical software (Cary, NC).

#### Results

##### $\text{IC}_{50}$ of EPI-HNE-4 on Active NE Excess Secreted in Sputum of Children with CF

Studies were performed on both whole sputum and soluble fraction of sputum from seven children with CF of various degrees of severity. Before being vortexed and centrifuged, whole sputum must be 4- to 8-fold diluted to obtain homogeneous extracts. Results summarized in Table 1 show that the initial NE activity in whole sputum ranged from 92–614  $\mu\text{g}/\text{ml}$  according to the degree of severity of the disease and that the EPI-HNE-4 inhibitor was able to effectively inhibit active NE excess in sputum from all children with CF. In most cases, especially in soluble fractions of sputum, the ratio  $f$  between measured  $\text{IC}_{50}$  and calculated  $\text{IC}_{50}$  was close to or equal to 1, reflecting optimal efficacy of the inhibitory capacity of EPI-HNE-4 in complex biologic fluids such as sputum from children with CF. However, in some severe cases, for which  $f > 1$ , the presence of large quantities of mucins or other components of sputum appeared to interfere with the accessibility of EPI-HNE-4 to the NE catalytic site.

##### Inhibitory Capacity of EPI-HNE-4 on Transmigration of Human Neutrophils Across Matrigel Membranes

Investigation of neutrophil chemotaxis in a Matrigel Invasion Chamber showed that neutrophil migration across the Matrigel membrane was barely detectable in absence of the chemoattractant ( $10^{-7}$  M FMLP), whereas marked transmigration ( $42 \pm 5$  neutrophils recovered per field) was dem-

TABLE 1  
*Inhibitory capacity (IC<sub>50</sub>) of EPI-HNE-4 on active NE excess secreted in sputum of children with CF*

Sputum No.	Initial NE $\mu\text{g/ml}$	Dilution Factor	Molar [NE] in Diluted Fraction	Measured IC <sub>50</sub> (M)	Calculated IC <sub>50</sub> (M)	<i>f</i>
1 sputum	92	4	$0.9 \times 10^{-6}$	$5 \times 10^{-7}$	$5 \times 10^{-7}$	1
1 soluble		4	$0.2 \times 10^{-6}$	$7 \times 10^{-8}$	$10^{-7}$	0.7
2 sputum	488	8	$2.4 \times 10^{-6}$	$2.3 \times 10^{-5}$	$1.2 \times 10^{-6}$	19
2 soluble		8	$2.3 \times 10^{-6}$	$1.8 \times 10^{-6}$	$1.2 \times 10^{-6}$	1.5
3 sputum	368	4	$3.6 \times 10^{-6}$	$6 \times 10^{-6}$	$1.8 \times 10^{-6}$	3
3 soluble		4	$1.4 \times 10^{-6}$	$10^{-7}$	$0.7 \times 10^{-7}$	3
4 sputum	332	4	$3.3 \times 10^{-6}$	$1.5 \times 10^{-6}$	$1.5 \times 10^{-6}$	1
4 soluble		4	$3.1 \times 10^{-6}$	$1.5 \times 10^{-6}$	$1.5 \times 10^{-6}$	1
5 sputum	614	8	$2.9 \times 10^{-6}$	$2.3 \times 10^{-6}$	$1.4 \times 10^{-6}$	1.6
5 soluble		8	$2.9 \times 10^{-6}$	$1.4 \times 10^{-6}$	$1.4 \times 10^{-6}$	1
6 sputum	132	4	$1.2 \times 10^{-6}$	$6 \times 10^{-7}$	$6 \times 10^{-7}$	1
6 soluble		4	$9.3 \times 10^{-7}$	$5 \times 10^{-7}$	$4.6 \times 10^{-7}$	1
7 sputum	307	4	$3.1 \times 10^{-6}$	$8 \times 10^{-6}$	$1.5 \times 10^{-6}$	5.2
7 soluble		4	$3.4 \times 10^{-6}$	$2.5 \times 10^{-6}$	$1.7 \times 10^{-6}$	1.4

*Definition of abbreviations:* CF, cystic fibrosis; NE, neutrophil elastase.

Factor *f* is the ratio between measured IC<sub>50</sub> and calculated IC<sub>50</sub> in diluted sputum or diluted sputum-soluble fractions. All experiments were carried out in triplicate. Values for each triplicate experiment were remarkably reproducible, and all mean values were associated with small standard deviations (< 10%).

onstrated when FMLP was added to the lower chamber. The addition of  $10^{-6}$  M EPI-HNE-4 inhibitor to the upper chamber markedly (by 81%) and significantly ( $P < 0.0001$ ) inhibited neutrophil migration across the basement membrane ( $8.2 \pm 1.7$  neutrophils recovered per field).

When neutrophil chemotaxis was investigated without Matrigel and in the presence of FMLP in the lower chamber, the number of neutrophils recovered was much higher ( $182 \pm 20$ ) than in the presence of Matrigel, and the additional presence of  $10^{-6}$  M EPI-HNE-4 inhibitor in the upper chamber slightly reduced neutrophil chemotaxis by 20% ( $145 \pm 18$ ) ( $P < 0.05$ ).

#### Early Pulmonary Response to Instilled Pure HNE or Soluble Fraction of Sputum (Containing Active NE)

We compared the pulmonary responses at 4 h after intratracheal instillation of saline solution (Series 1), 150  $\mu\text{g}$  of pure HNE (Series 2), or soluble fraction of CF sputum containing 120  $\mu\text{g}$  of free active HNE (Series 3).

Instillation of pure HNE induced severe hemorrhage in the lungs as evidenced by scattered foci of hemorrhagic alveolitis in the whole lung (Figure 1B) compared with control lungs (Figure 1A) and by the 5.5-fold increase in hemoglobin in BAL ( $992 \pm 98$  versus  $172 \pm 15$  mg/l) (Figure 2). Acute alteration of alveolar permeability was also observed as evidenced by the 8-fold increase in BAL albumin ( $161 \pm 18$  versus  $20 \pm 3$  mg/l). A free NE activity ( $2.3 \pm 0.2$  mOD/min/50  $\mu\text{l}$ ) persisted in BAL, whereas no free NE activity was detected in the control group. A slight but significant ( $P < 0.0001$ ) neutrophil influx into air spaces was also observed ( $2.7 \pm 0.3$  versus  $0.4 \pm 0.2 \times 10^5$  neutrophils). However, macrophages remained the predominant cells (81% of all cells recovered in BAL).

By contrast, intratracheal instillation of CF sputum soluble fraction (Figure 3) did not induce any hemorrhage or

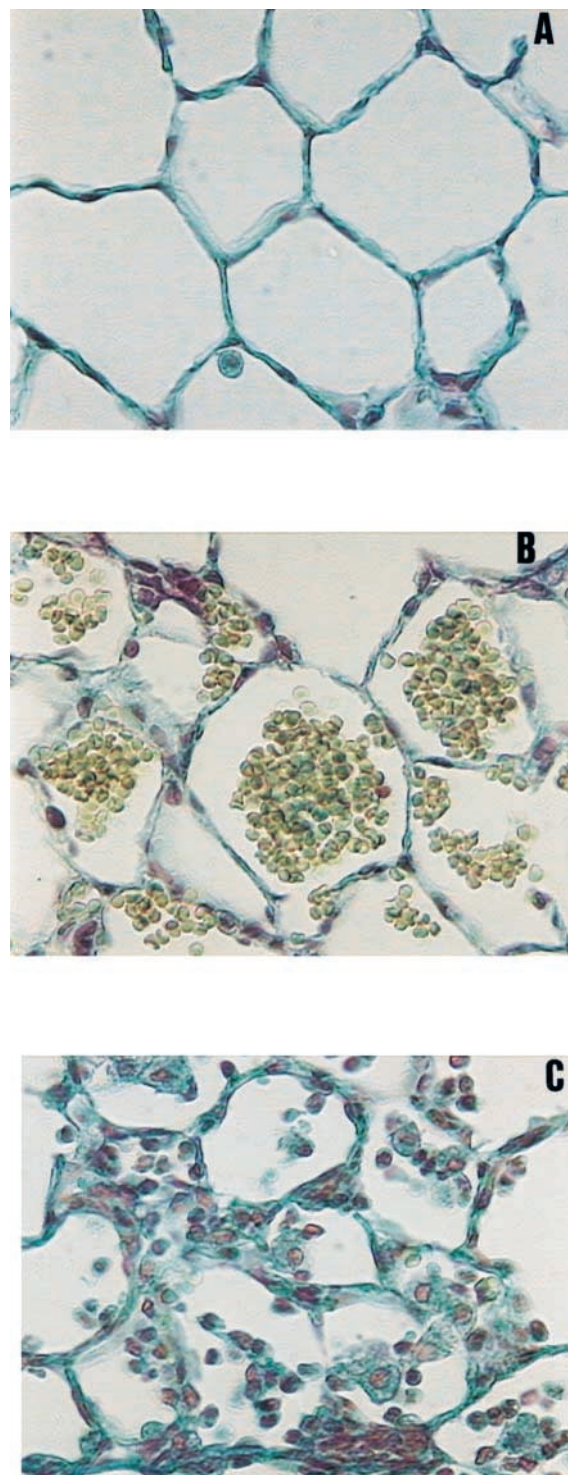
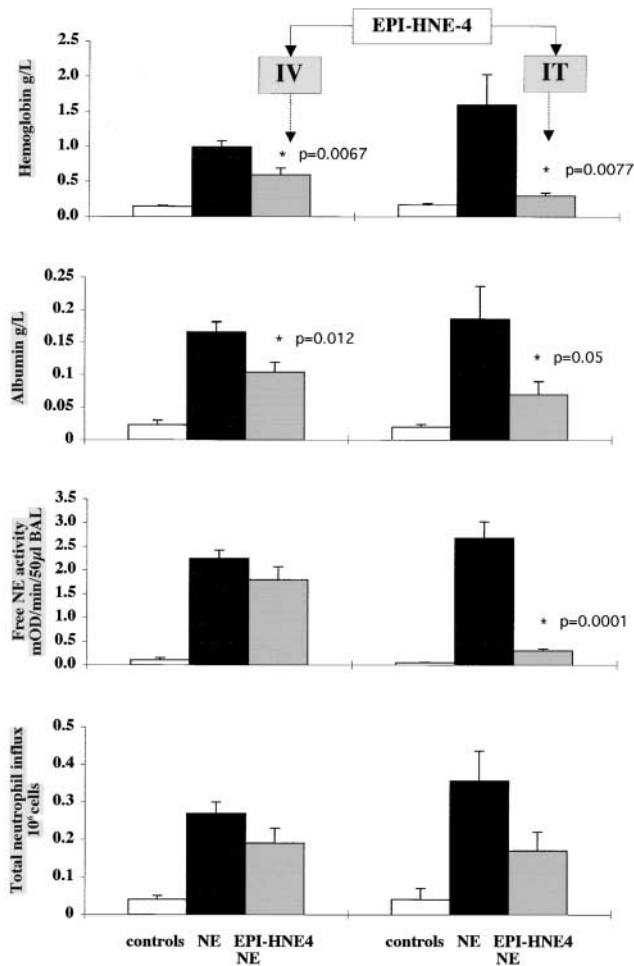


Figure 1. Histologic studies of the lung ( $G \times 40$ ) (20  $\mu\text{m}$ ). (A) Histologic views of control lungs. (B) Histologic views of acute lung injury induced by pure HNE. Note the scattered foci of hemorrhagic alveolitis in the lung at the fourth hour after instillation of NE. (C) Histologic views of acute lung injury induced by sputum soluble fraction. Note the scattered and acute neutrophil influx in air spaces and septal structures at the fourth hour after instillation of sputum soluble fraction.

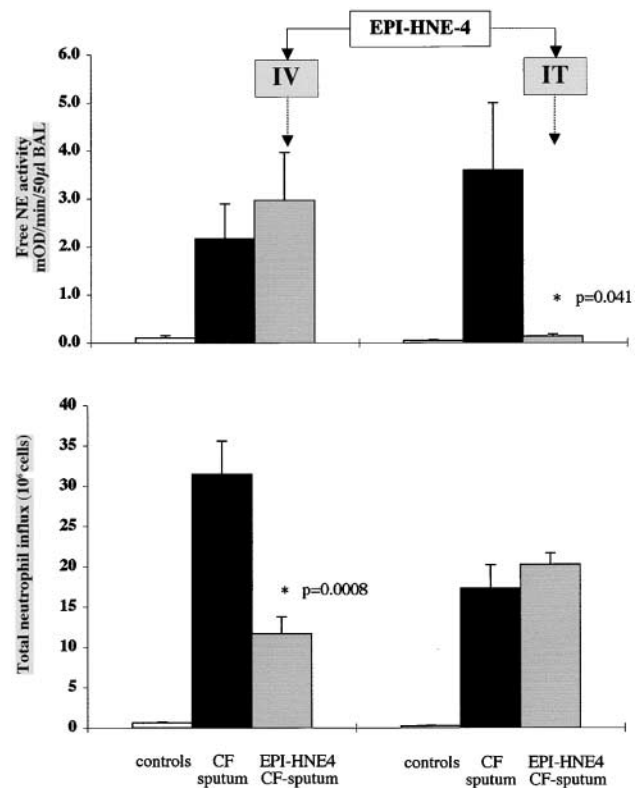


**Figure 2.** Protection against pure HNE-induced acute lung injury by intravenous or intratracheal pretreatment with EPI-HNE-4. In the two series, instillation of pure HNE (150 µg per rat) induced, 4 h later, a marked, reproducible, and highly significant increase ( $P < 0.0001$ ) in hemoglobin, albumin, free NE activity, and neutrophil influx in the BAL of rats. Intratracheal EPI-HNE-4 pretreatment (250 µg per rat) 5 min before instillation of pure HNE markedly and significantly ( $P < 0.001$ ) reduced hemoglobin, albumin, and free NE levels but induced only a nonsignificant ( $P = 0.07$ ) reduction of neutrophil influx. Intravenous pretreatment (3 mg per rat), 15 min before instillation of pure HNE, significantly reduced hemoglobin and albumin level, but less effectively than intratracheal pretreatment.

serum albumin leakage but produced massive chemoattraction of neutrophils ( $32 \pm 3$  versus  $0.4 \pm 0.2 \times 10^6$ ), which became the predominant inflammatory cells in the air spaces (74% of all cells recovered in BAL). A free NE activity, of the same order as described above ( $2.5 \pm 0.5$  mOD/min/50 µl), was also demonstrated in the BAL. When compared with controls (Figure 1A), histologic studies showed acute neutrophil influx into air spaces and septal structures (Figure 1C).

#### Protection against Acute Lung Injury by Intravenous or Intratracheal Pretreatment with EPI-HNE-4

In the experimental model of acute lung injury induced by intratracheal instillation of pure HNE (150 µg per rat) (Fig-



**Figure 3.** Protection against sputum-soluble fraction-induced acute lung injury by intravenous or intratracheal pretreatment with EPI-HNE-4. In the two series, instillation of sputum-soluble fraction (containing 120 µg NE) induced, 4 h later, a marked increase in active NE and massive neutrophil influx in the BAL of rats. Intratracheal EPI-HNE-4 pretreatment (250 µg per rat), 5 min before instillation of sputum-soluble fraction, markedly and significantly ( $P < 0.05$ ) reduced only active NE, whereas intravenous pretreatment (3 mg per rat), 15 min before instillation of sputum-soluble fraction, markedly and significantly ( $P < 0.001$ ) reduced only massive neutrophil influx.

ure 2), intravenous EPI-HNE-4 treatment (3 mg per rat) administered 15 min before NE significantly decreased hemorrhage and serum albumin leakage by 47 and 43%, respectively, but did not appear to significantly reduce neutrophil influx or residual free NE activity in BAL. Intratracheal EPI-HNE-4 treatment (250 µg per rat), administered 5 min before NE, significantly prevented hemorrhage, serum albumin leakage, residual free NE activity, and moderate neutrophil influx in BAL by 94, 67, 89, and 62%, respectively.

In the experimental model of acute lung injury induced by intratracheal instillation of CF sputum-soluble fraction (120 µg of free NE per rat) (Figure 3), intravenous EPI-HNE-4 treatment (3 mg per rat) administered 15 min before NE significantly inhibited acute neutrophil influx in BAL by 64%, whereas no significant inhibitory effect was observed on the residual level of free NE activity in BAL. By contrast, intratracheal instillation of EPI-HNE-4 (250 µg per rat) administered 5 min before NE totally blocked the residual free NE activity in BAL, whereas no significant inhibitory effect was detected on acute neutrophil influx in BAL.

### Dose-Dependent Inhibitory Effect of Instilled EPI-HNE-4 on Pure HNE-Induced Acute Lung Injury

Intratracheal instillation of EPI-HNE-4 induced a dose-dependent reduction of the severity of pure HNE-induced lung injury (Figure 4). As described above, the highest dose, corresponding to a 6.6-fold molar excess over NE, completely blocked hemorrhage, serum albumin leakage, and free residual NE activity in BAL and very effectively reduced the moderate neutrophil influx in BAL by 83%. The lowest dose, corresponding to a 2.6-fold molar excess over NE, was still able to significantly inhibit hemorrhage, serum albumin leakage, and free residual NE activity in BAL by 51, 49, and 60%, respectively, and tended to decrease the moderate neutrophil influx by 35%.

### Discussion

The present data demonstrate that, *in vitro*, a new, extremely potent ( $K_i = 2$  pM) HNE specific inhibitor (EPI-HNE-4) is able to effectively inhibit the free NE present in the sputum of children with CF and almost completely block the FMLP-induced migration of human purified neutrophils across a Matrigel basement membrane. This study also demonstrates that intratracheal and intravenous injections of EPI-HNE-4 are able to reduce the two types of acute lung injury induced in the rat by instillation of either pure HNE or CF sputum-soluble fraction.

The initial aim of our study was to test whether EPI-HNE-4 was able to inhibit the free NE activity present in an environmental biologic context as complex as sputum from children suffering from CF. We hypothesized that high levels of mucins, oxygen-free radicals, bacterial components, and various mediators in bronchial air spaces during CF might affect or impede effective accessibility of EPI-HNE-4 to the catalytic site of free NE. The assays performed on both sputum and related soluble fraction from children with CF clearly showed that, in most cases, EPI-HNE-4 was able to inhibit the high levels of free NE activity present in the sputum (92–614  $\mu\text{g}/\text{ml}$  sputum) with an  $\text{IC}_{50}$  equal to or close to that obtained with the same concentration of HNE in saline solution. Moreover, as stated above, this inhibitory capacity is very specific for NE: an inhibitory capacity was never detected against *Pseudomonas aeruginosa* elastase, cathepsin G, or proteinase 3 (data not shown). Three of the seven samples of whole sputum from children with CF and one related sputum-soluble fraction required higher molar levels of EPI-HNE-4 over HNE to effectively inhibit free NE, suggesting that mucins might interfere with the accessibility of the NE inhibitor to the NE catalytic site. All these data suggest a possible therapeutic approach based on chronic administration of very small amounts of aerosolized EPI-HNE-4 in children with CF, which might effectively attenuate lung structural and functional damage induced by excessive levels of NE activity. These lesions are thought to be at least partially responsible for progressive destruction of small bronchioles and subsequently larger airways observed in CF (1–3, 5–7). Also, as previously described, excess NE is known to promote epithelial damage, vascular hyperpermeability, mucus hypersecretion, mucous gland hyperplasia, degradation of most of the extracellular matrix components, and

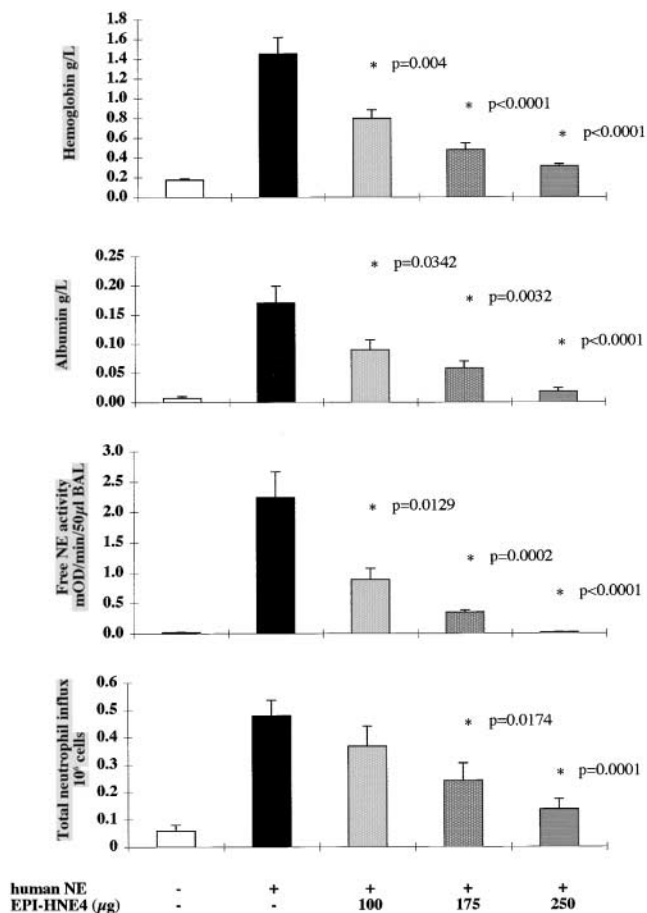


Figure 4. Dose-dependent inhibitory effect of instilled EPI-HNE-4 on acute lung injury induced by pure HNE. As expected and as shown in Figure 2, instillation of pure HNE (150  $\mu\text{g}$  per rat) induced, 4 h later, a marked, reproducible, and highly significant increase ( $P < 0.001$ ) in hemoglobin, albumin, free NE activity, and neutrophil influx in the BAL of rats. Intratracheal EPI-HNE-4 pretreatment 5 min before instillation of pure HNE reduced all these effects in a dose-dependent fashion.

generation of chemotactic peptides and cytokines, as well as reduce mucociliary clearance (7–16).

Uncontrolled excessive secretion of NE to levels higher than those of its natural inhibitors, namely SLPI and  $\alpha$ 1-proteinase inhibitor, in the sputum of children with CF reflects chronic neutrophil-dominated inflammation on the epithelial surface of the airways because intense neutrophil migration into sites of inflammation is a critical feature of host defense against microorganisms such as *P. aeruginosa* and *Staphylococcus aureus*, the most frequent colonizing bacterial pathogens in CF (25). In response to bacterial infections or inflammatory stimuli, neutrophils adhere to the endothelial surface of postcapillary venules, cross the endothelium via interendothelial cell junctions, and penetrate across the basement membrane, leaving the vascular system to accumulate at the site of inflammation. Neutrophil transmigration is thought to be at least partly dependent on the degradation of basement membrane components by neutrophil enzymes, elastase, and gelatinase B stored in specific granules (26–28). Our recent studies (23)

strongly suggest that HNE may contribute to the human neutrophil migration process by activating progelatinase B. The present study, evaluating human neutrophil migration in response to  $10^{-7}$  M FMLP chemoattractant, using the same experimental procedure across a Matrigel basement membrane coated on a filter in a Matrigel Invasion Chamber, clearly demonstrates that EPI-HNE-4 is able to effectively inhibit neutrophil transmigration by 80%. In the absence of Matrigel and in response to FMLP, only a slight, nonsignificant inhibitory effect on neutrophil chemotaxis was observed, suggesting that NE would be more involved in the proteolysis of Matrigel components and therefore in active neutrophil transmigration through matrix components than in FMLP-induced neutrophil chemotaxis *per se*. However, by using strains of mice deficient in NE or gelatinase B by targeted mutagenesis, other studies suggest that neither neutrophil elastase nor gelatinase B are required for successful neutrophil transendothelial migration (29) and that gelatinase B is not required for neutrophil emigration in the lungs, peritoneum, and skin (30). The lack of inhibition of neutrophil transendothelial migration by SLPI also suggests that NE does not have a direct role in digesting basal lamina during neutrophil extravasation (31). Taken together, these results raise the question about the exact role of proteinases in neutrophil migration. Our *in vitro* studies tested whether the EPI-HNE-4 inhibitor may also control *in vivo* neutrophil influx as well as the excessive HNE level in air spaces during acute lung injury.

In this context, we investigated the comparative potential of EPI-HNE-4, when administered intravenously or intratracheally, to mitigate the observed lung injury induced by either pure HNE or by soluble fraction of CF sputum containing similar amounts of active HNE.

Intratracheal instillation of pure HNE at the dose of 150  $\mu$ g per rat induced severe hemorrhage 4 h later, as previously described by several authors (20, 25), together with a marked reduction of alveolocapillary permeability and a slight but significant 7-fold increase of neutrophil influx into the air spaces, whereas residual active NE, corresponding to approximately 2% of total dose instilled, persisted in the air spaces, indicating that the antielastase pattern was not sufficient to prevent HNE-induced lung damage.

At the same time point (4 h after instillation), a similar residual active NE was still present in the air spaces of rats treated with CF sputum-soluble fraction containing 120  $\mu$ g of active NE. By contrast, in the later experimental model, no hemorrhage or alteration of alveolocapillary permeability was observed, strongly suggesting that components of CF sputum-soluble fraction may act by reducing some of the toxic effects of NE. Also at this time point, a much greater inflammatory reaction was characterized by a massive 80-fold increase of neutrophil influx. We deliberately focused our study exclusively on this "4 h" time point on the basis of data previously reported by Rees and colleagues (20). These authors investigated neutrophil infiltration in the rat at several time points (2, 4, and 6 h) in response to intratracheal instillation of CF sputum-soluble fraction, and their results showed that, for instillation of 150  $\mu$ l of CF sputum-soluble fraction (containing 130  $\mu$ g of NE/100  $\mu$ l), the BAL neutrophil influx was markedly in-

creased after 4 h and further increased at 6 h. Because our own experimental data clearly demonstrated an acute 80-fold increase of the BAL neutrophil influx by 4 h after CF sputum intratracheal instillation, this time point appeared to be particularly relevant to reliably evaluate the protective effect of EPI-HNE-4 inhibitor on neutrophil influx into the airways. Previous observations suggested a self-perpetuating inflammatory process on the CF bronchial surface, in which NE released by neutrophils could induce secretion of interleukin (IL)-8 by the bronchial epithelium, resulting in further recruitment of neutrophils to the bronchial surface (15). Our comparative data, indicating a slight neutrophil influx in response to the instillation of pure HNE versus a massive neutrophil influx in response to the instillation of CF secretions, strongly argue in favor of the presence of reagents such as IL-8 in CF sputum-soluble fraction to investigate mechanisms of massive neutrophil recruitment in rat lungs. For a similar instilled dose of HNE (120–150  $\mu$ g per rat) and at the same study time (4 h after instillation), the two models therefore appeared somewhat different and allowed us to test the potential protective effect of EPI-HNE-4.

Our results show that (i) intratracheal treatment of rats with EPI-HNE-4 (250  $\mu$ g per rat) can significantly protect them against hemorrhage, alveolocapillary permeability, and active NE excess in air spaces induced by instillation of pure HNE (150  $\mu$ g per rat) with greater efficacy than systemic treatment (10 mg/kg) and (ii) this protective effect remains effective even at lower doses, according to a dose-dependent pattern. Concurrently, intratracheal treatment of the rats with EPI-HNE-4 (250  $\mu$ g per rat) also effectively protected them against active NE excess in the air spaces induced by CF sputum-soluble fraction instillation (containing 120  $\mu$ g of free NE equivalent per rat).

Regarding the protective inhibitory effect of EPI-HNE-4 against neutrophil influx in the CF sputum-induced acute lung injury, systemic treatment of the rats with EPI-HNE-4 effectively protected them against massive neutrophil influx, whereas intratracheal treatment was completely devoid of any protective effect. Restrictive modulation of neutrophil recruitment *in vivo* by intravenous administration of higher doses (30 mg/kg) of truncated secretory leukoprotease inhibitor has also been observed during bleomycin-induced pulmonary fibrosis in hamsters, but to a lesser extent (32). On the basis of our results, we can propose that, in the CF sputum-induced acute lung injury model, systemic administration of EPI-HNE-4 could directly prevent transmigration of actively recruited neutrophils from the pulmonary microcirculation, whereas intratracheal administration is unable to access to vascular transmigrating neutrophils.

In conclusion, taken together, our data clearly demonstrate that, *in vitro*, EPI-HNE-4 is able to effectively inhibit the high levels of active NE present in a medium as complex as sputum from children with CF, as well as migration of chemoattractant-purified human neutrophils across a basement membrane. Intratracheal administration of EPI-HNE-4 also effectively protected the lung from hemorrhage, serum albumin leakage, and active NE excess in air spaces, while intravenous administration effectively prevented massive neutrophil influx, strongly suggesting that combined aerosol and

systemic administration of EPI-HNE-4 would be beneficial in the treatment of CF.

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